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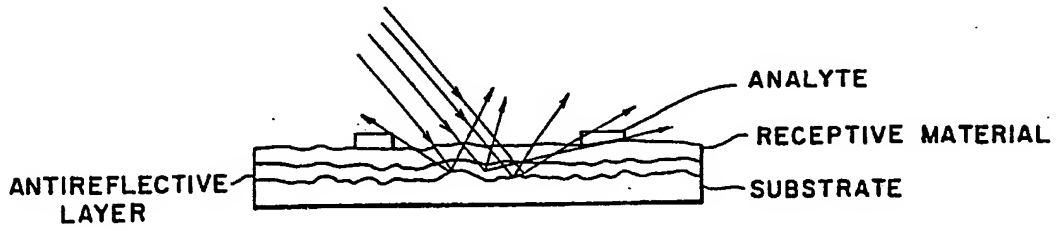

(57) Abstract

A method and apparatus for the detection of the reaction between receptive material and an analyte, whereby the reaction itself generates an optical interference signal. The assay technique is based on the affinity of the analyte for the receptive ligand. The resultant reaction between the two specific binding partners provides for qualitative and/or quantitative detection of the chemical, biochemical, or biological analyte in the sample. The assay apparatus is comprised of (a) a substrate that can be coated with anti-reflective optical layers; (b) a thin film of anti-reflective material capable of being activated to bind the receptive material; (c) a layer of receptive material capable of interacting with the species to be assayed. The components a-c result in a pre-formed surface that is otpically active with respect to radiation at least over a certain band of wavelengths. The assay technique comprises the steps of (d) contacting the pre-formed surface with the sample; and (e) observing the optical properties of the pre-formed surface in order to visually detect a change in the optical properties of the pre-formed surface as a result of the binding of the analyte within the sample to the receptive material.

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APPARATUS FOR DETECTION OF AN IMMOBILIZED ANALYTE

FIELD OF THE INVENTION

This invention relates to the field of biochemistry and chemistry, with applications in such areas as immunoassay, nucleic acid sequence analysis, clinical chemistry, enzyme assay, and the study of ligand/anti-ligand binding pairs such as toxin-receptor or hormone-receptor combinations, etc. This invention is a solid phase assay using direct physical detection methods based on optical interference properties. In a solid phase assay, the receptive material is immobilized on a coated support surface. The immobilized receptive material is exposed to a fluid that may contain the analyte, which is a molecule of interest capable of binding to or reacting with the receptive material. The analyte can be either organic or inorganic. In general, the roles of the receptive material and the analyte in the assay can be reversed.

PRIOR ART

Assays exist in the art for monitoring reactions between homologous couples such as antigen-antibody binding, hormone-receptor binding, enzyme assays, and nucleic acid hybridization. The critical part of assay design in each case is deciding how the signal will be generated. Colored reaction products, biological activity at the cellular level, and radioactive, fluorescent, or luminescent tags covalently bound to one of the reacting species have all been used. Two familiar examples in the field of immunoassay are enzyme-linked immunosorbent assay (ELISA) and radioimmunoassay (RIA). In all of these methods, the experimenter does not actually detect the binding of the two molecules, but some secondary activity related to the binding reaction. Most of these techniques require special steps for signal generation, adding to labor costs, and often require the

use of hazardous materials and expensive instrumentation.

A more advantageous means of detecting binding or reaction between two species of biological molecules is to detect a change in some physical property that is a direct result of binding or reaction. For example, if the ligand is present as a thin film immobilized on a support, then any method that can measure a change in thickness or mass of the biological thin film after binding or reaction with the analyte would be a means for direct physical detection. This invention provides such a method, based on the principles of optical interference in thin films.

Optical detection techniques have existed in biochemistry since the middle part of this century, especially in the area of immunoassay. All of these techniques utilize: a solid support or substrate; intermediate layers that may be necessary for generating the signal; a thin film (usually monomolecular) of a biologically active molecule, the ligand; and an optical set-up comprised of a source of radiation, a means for setting the angle of incidence, if necessary, and a means of detection. The various optical assays measure different properties of light, use different substrates, and are of varying degrees of utility in different situations (research laboratory vs physician office laboratory vs home or field use).

The first solid-phase immunoassay using optical detection was based on the Langmuir-Blodgett technique. The technique provides a means for spreading monolayers of amphipathic molecules onto the surface of water, then transferring these films to a glass or metal substrate. Multilayer films of fatty acids can be built up on metal by repeated dipping of the substrate through the monolayer, which could then be used as supports for monolayers of proteins (Blodgett, 1934). In the

published immunoassay (Langmuir and Schaefer, 1937), a barium stearate film was built up to a thickness of about fifty monolayers on a chromium plated glass slide. The barium stearate film was then coated with a layer of diphtheria toxin, which added 3.6 nm to the thickness of the film. The ligand-coated slide was then dipped into a solution containing anti-toxin antibodies. After incubation with the antibodies, the thickness of the film increased a further 7.5 nm. In this experiment, the thickness changes were measured by illuminating the slide with s-polarized light at large angles of incidence and measuring the reflectance minimum. The optical signal is sensitive enough to detect thickness changes as small as 1 Angstrom.

Although very sensitive tests are possible, assays based on the Langmuir-Blodgett technique are no longer of practical importance in biochemistry because of serious disadvantages. The technique is very difficult and time-consuming, and requires reagents, especially water, of very high purity.

The principle of optical interference through the superposition of light waves with varying phase relationships and amplitudes has been used to monitor biological reactions. The first optical assays using interference colors as a means of detection used metal or metallized substrates, and the ligand molecule was simply absorbed to the surface. For example, Adams, et al. (1973) used anodized tantalum with a tantalum oxide layer, and Giaever (1974) U.S. Pat. 4,054,646, used indium coated glass slides. These substrates have disadvantages in that they are difficult to handle. The metal surfaces are also disadvantageous from the chemistry point of view. Metal ions can leach from the surface and interfere with the chemistry of binding between the ligand and the analyte. Furthermore, the

surfaces of such substrates are not as versatile for organic, covalent linkages as a silica or glass surface.

Two more recent inventions use reflectance or optical interference effects to detect changes in thin organic films due to binding of the analyte. One method monitors changes in thickness and the other looks at the change in polarizability of immobilized material. Arwin and Lundstrom (1985) use a method that is reminiscent of the original work by Langmuir, Blodgett, and Schaefer. They used a silicon substrate, with a thermally grown silicon dioxide layer, about 10 nm thick. The surface was made hydrophobic with the use of dichlorodimethylsilane, and in the published example, human serum albumin was adsorbed onto the hydrophobic surface, presumably as a monolayer. The invention exploits the different reflectance properties of p-polarized and s-polarized light. At the Brewster angle, which is determined by the refractive indices at the boundary between two media, no p-polarized light is reflected. When the substrate is an absorbing material and the medium is transparent as in this case, the reflectance minimum is shifted to the so-called pseudo-Brewster angle. At this angle of incidence, p-polarized light has measurable, but minimal reflectance. When the thickness of the dielectric layer increases, for instance due to antibody binding to antigen, there is an increase in reflectance at the pseudo-Brewster angle. The method is simple in concept, but requires some precise instrumentation in practice. First, a polarizing means must be used to obtain p-polarized light. For use with a silicon substrate, the incident light must be further processed with a filter to eliminate red wavelengths. Other substrates may require monochromatic or near-monochromatic light. Two photodiodes are required: one to measure reflected intensity and one to measure the intensity of the light source. An electronic unit

containing an amplifier and a display are also needed. Most importantly, the angle of incidence must be measured very precisely, so all components and the sample must be mounted in a machined housing. This published account focused specifically on an immunoassay format.

Another related method is disclosed by Nicoli, et al. in U.S. Patent 4,647,544. This patent discloses a method for immunoassay using the principle of optical interference. The interference effect used in this invention is a change in light intensity at various point in space rather than a change in interference color. In particular, this method detects constructive interference of light at Bragg scattering angles. A crucial part of this invention is the preparation of the surface. The ligand (e.g. antibody) is applied as a regular array on the substrate. The preferred embodiment is to apply the ligand in strips of width w and center-to-center spacing d, where d is greater than w. Both d and w are of microscopic dimensions. The regular array acts as a diffraction grating, and maximal scattering occurs at angles O_S, where: $d \sin O_S = m\, l_S$ ($m = 1,2,3,\ldots$). The important physical property measured in this scheme is a change in polarizability relative to the medium. When an antigen binds to the immobilized antibody, there is an increase in polarizable mass at regular intervals in the array, and the intensity of light at angles O_S increases as well. As this method of detection observes second order effect, there is a reduction in assay sensitivity relative to other interference phenomena.

In another embodiment, they coat an actual diffraction grating with the ligand and detect Bragg scattering in the same way. In both embodiments, the peaks of scattered light can be detected in either the reflected or transmitted light depending on the nature of the substrate.

As in the method of Arwin and Lundstrom, special light sources and optical equipment are necessary to detect the interference effects. Since only intensity changes due to optical interference are being detected, a monochromatic light source must be used. In the disclosed example, a He/Ne laser served as the light source. Detection at the scattering angle is achieved with a device such as a photomultiplier tube or solid state photodiode.

The technology most closely related to the present invention was first disclosed by Nygren, et al. (1985) in U.S. Patent 4,558,012. This patent covers a method and a device for optical interference assays that allow for direct detection of results using a non-metal substrate and a dielectric film. The dielectric film is made up of a layer of SiO_2 (1.46) is very close to that for most organic molecules (n = 1.5), so that the two substances can function as a single layer for optical purposes. The thicknesses are chosen such that the entire dielectric film can act as a single layer anti-reflection coating, optimized for a wavelength present in the polychromatic incident light, giving the unreacted slide a characteristic interference color. When the analyte binds with the ligand, the optical pathway changes, giving a new wavelength minimum in the reflected light and a new visible interference color. Use of a polychromatic light source means that an interference color is seen in the reflected light rather than interference fringes or an intensity change. When the analyte binds with the ligand, the optical pathway changes, giving a new wavelength minimum in the reflected light an a new visible interference color. A similar technology is described in these Teijin patent applications: Japan Patent Application SHO 61-222057, and SHO 61-222058; and the Teijin European patent application

87113842.6 shows a method to enhance the visible interference signal.

Solid phase assays occasionally use surfaces that are not perfectly planar. Those assays that start with a polymeric surface are at least microscopically irregular. Surfaces that are etched or patterned in a specific array are critical to the detection method disclosed by Nicoli et al. (U.S. Patent No. 4,647,544).

Methods for physically roughening or etching the surface of a silicon substrate are known in the electronics and semiconductor industries. Some of these methods are also applicable to glass and silica surfaces.

Anisotropic etching of silicon, gallium arsenide, and germanium provides means for introducing structure or texture onto the surface of these substrates. One common etch method utilizes potassium hydroxide or sodium hydroxide. The etch rate and depth can be controlled by varying the strength of the alkaline solution and using selected additives.

For example, Stoev and Petkanov (1982) describe the use of n-propanol and hydrazine in KOH solutions to decrease the etch rate and reduce defects in the manufacture of VMOS structures. Erdman, et al. (Ger Offen 2,245,809) describe the use of H_2O_2, K_2CrO_4, and tetrahydrofurfuryl alcohol as additives in alkaline etching solutions. Another aqueous etching system described by Petkanov for production of VMOS structures is a combination of ethylenediamine-pyrocatechol-water.

Dry etching procedures are also available for anisotropic etching. Japanese patent JP 81,144,541 teaches the use of an etchant gas containing C_2F_5Cl and CF_2. Barkanic, et al. (1987) use mixtures of NF_3 in CF_3Cl, CF_2Cl, and CF_3Br in a plasma generator. In this case, portions of the surface can be masked with SiO_2.

It is an object of this invention to provide a biochemical assay using optical detection, specifically the generation of interference colors, to monitor binding and/or reaction between two molecular species--the ligand and the analyte. The device is an interference slide comprised of: a first layer, the substrate, of high refractive index; (optionally) one or more optical layers of lower refractive index and varying thickness; a bound layer of receptive material such as organic ligand molecules.

It is an objective of one of the embodiments of this invention to provide a biochemical assay capable of selectively attaching an analyte and generating a corresponding binding signal which need not be visible but is capable of instrumented detection.

A further objective of this invention includes an improvement in optical interference assays in the form of an irregular surface which produces a diffuse reflection. The surface imperfections are not regularly spaced as in a diffraction grating, and the variations in height can be in the range from hundreds of nm to about 100 um.

The irregular surface creates three main advantages. The first of these is increased surface area available for coating with receptive material, and a concomitant increase in reaction speed and/or sensitivity of the assay. The second advantage is that the interference colors are seen in diffuse rather than specular reflection. In contrast to a diffuse reflection when interference colors are seen against a specular reflecting surface, images of distant objects are also visible in the reflected light. These images can confuse the viewer or even cover up spots of color, especially if the color is faint and the assay is near the limit of detection. The third advantage is that a diffuse reflection allows the color change to be visible

over a broad range of angles of incident light. Specular reflections, in contrast, are dependent on the angle of incident light and if the article is incorrectly positioned with reference to the light source the color change becomes indistinct.

Furthermore, among the objectives of the present invention is to provide a novel method of making and performing an immunoassay that can be used in laboratories, and is simple and inexpensive enough for use in the consumer's home.

A further mission of this device is to provide a versatile assay. This invention can be made from a wide variety of substrates, anti-reflective materials, and receptive materials. The proper selection of material components allows the test to be formatted to generate the type of signal the end user requires. For example, most home use assays simply require qualitative (positive vs. negative results) but for use in the practitioner's office often a more quantitative result is required to aid diagnosis or monitor therapeutic efficacy; the various embodiments of this invention provide for this type of format versatility.

SUMMARY OF THE INVENTION

The present invention includes novel compositions and novel articles for the direct selective absorption, adsorption, covalent binding or attachment by whatever mechanism, of an analyte, specific to the receptive material bound to the surface of the apparatus for the purpose of identification or quantitation of the analyte. The present invention in its broadest sense provides a method of detection of an analyte without the use of labels such as radioactive enzyme, fluorescent, or luminescent conjugates and the like, i.e. using unlabelled detection materials. This method of detection comprises the steps of depositing one or more anti-

reflective coatings onto a substrate material, covalently binding or adsorbing, by whatever mechanism affixing, receptive material specific to the analyte of interest to the top layer of anti-reflective material; contacting the receptive material with a fluid containing the corresponding analyte of interest, and then examining the diffuse reflection produced by the coated article to determine whether a colored spot or other suitable optical signal appeared. In the first embodiment of this invention the substrate has an irregular surface, therefore the reflection is diffuse. In the second embodiment, however, the reflection is specular, then smoked glass or textured plastic, or any other light reducing, light scattering, light diffusing, or light attenuating, or light modifying, etc. material is placed over the article to produce a non-specular reflection. In the third embodiment of this invention the substrate is not necessarily coated with anti-reflective material and the device is designed to generate an optical signal which can be detected by an instrumented technique. Major drawbacks of many specular assays intended for use as non-instrumented visual tests are namely that the viewing often must be done through polarizers or filters, and that the assay's sensitivity is partially dependent on the angle of incidence of the light. For example, a substrate with a refractive index of 2.25 coated with an anti-reflective layer with a real refractive index of 1.50 has a lack of sensitivity when the incident light is 30 degrees or more from perpendicular incidence (T. Sandstroem, M. Stenberg, and H. Nygren, Applied Optics, Vol. 24, pg. 472-479 [2/15/85]). It is unique and surprising to find that an irregular substrate, or light diffusing material, placed over the article, would create a diffuse reflection that would reduce or eliminate the incident light angle dependence of the article and still reveal a clear signal, i.e. a change in interference

color. The present invention employs a method and article that produce a diffuse or non-specular reflection which is a substantial improvement over the assay detection methods previously known.

According to the preferred practice of this invention, a substrate is selected to have some of the various attributes described herein. The substrate can be formed of a reflective material such as polished silicon, polished metals, etc., or with little difficulty the assay can be performed on a transmissive substrate such as certain plastics, glass, quartz, etc. The substrate may be a solid support, a flexible support, a pellicle or a gel. Examples of some suitable materials for making a substrate are metal, quartz, plastic, silicon, non-metals or functionally equivalent materials capable of having anti-reflective material coated onto the surface.

A flexible support is especially useful for articles that are marketed as home test kits; because when shipped these articles do not require special handling or expensive protective packaging. Examples of some of the materials which can be suitable for forming flexible supports are various forms of plastics, or malleable metals, etc. The substrate can also be a pellicle which is both lightweight and flexible, or the substrate can be formed of a gel-like substance. Furthermore, the substrate can be any substance onto which is coated a layer of material which is capable of receiving an anti-reflective coating and meets the criteria hereinafter described. The critical criterion for the choice of substrate material is namely that it is capable of being coated with an anti-reflective material, and second is that the refractive index of the substrate is preferably approximately equivalent to the square of the refractive index of the material directly above it (although a wide variety of ratios between the

refractive indices of the substrate and the material directly above it can be utilized within the scope of this invention). The second criterion is important for signal generation; the interference color produced by the pre-formed article and the interference color produced by the reacted article should be visually contrasting to yield a highly visible signal.

According to the preferred practice of this invention the substrate has an irregular surface. The surface imperfections are not regularly spaced and the variations in height are on the order of 200-300 nanometers to about 100 micrometers. Methods for generating an irregular surface include anisotropic and isotropic etching of the surface or in some cases the nature of the substrate material renders the surface irregular. However, it should be understood that some of the embodiments of this invention utilize a smooth substrate surface as the preferred substrate.

After selection of the appropriate substrate an anti-reflective coating is affixed to the substrate. The anti-reflective coatings are deposited onto the surface of the substrate by known coating techniques, for example, by sputtering or by vapor phase deposition in a vacuum chamber. Material useful as anti-reflective coatings have four attributes, namely the material is clear and essentially colorless at the thickness utilized, stable at room temperature, sufficiently stable to withstand the deposition techniques, and by definition capable of suppressing reflective light when coated. Anti-reflective material serves two functions, first to produce an interference color, and second to provide a top layer onto which receptive material can be affixed in such a manner as to maintain the receptive material's capacity to selectively adsorb or bind any analyte of interest present in the fluid to be contacted with the apparatus.

The anti-reflective material in this invention produces an interference color by destructive interference of certain wavelengths of light; for example, an anti-reflective layer of silicon monoxide coated at a thickness of 600 Angstroms on a silicon substrate produces a bluish interference color, silicon dioxide on a similar substrate and at a similar thickness produces a gold color.

The thickness of the coated anti-reflective material is dependent on whether monochromatic or polychromatic light will be utilized to radiate the surface of the assay apparatus, and the degree of sensitivity desired in the assay. The greatest reduction in reflection occurs for an anti-reflective coating having a thickness of approximately one-fourth of the wavelength of light in the medium, and a refractive index that is the square root of the product of the indices of the media directly above and directly below it.

An article optimized to be radiated with polychromatic light should have an AR coating of a thickness of a quarter of a wavelength of the selected wavelength. If the article is to be radiated with monochromatic light and a high degree of sensitivity is desired, then the apparatus should be coated with one or more anti-reflective layers on the order of several wavelengths optical thickness. Using this thickness of anti-reflective layers provides the article with an enhanced finesse, by enhancing the wavelength selectivity of the interference effect. Thicker anti-reflective layers yield increasingly narrow wavelength ranges corresponding to the allowed reflected or transmitted light. For example, if the present invention was coated with a thick anti-reflective layer (e.g. 1 um) and irradiated with a fixed wavelength (e.g. 6328 Angstroms from a HeNe laser) an extremely small change in the

thickness of layers on the test device will produce a significant change in the wavelength of the reflected or transmitted light.

A reflective substrate coated with an optical thickness of one to several wavelengths of anti-reflective material may utilize the same optical principles as a Fabry Perot interferometer. The anti-reflective materials act as a cavity with a reflecting top and bottom surface that allows only certain optical wavelengths to exist therein. The reflected radiation from this article varies strongly as a function of wavelength. The thicker the anti-reflective layer or layers, the higher the Q of the cavity, and the narrower the band of allowed wavelengths will be, thus increasing the sensitivity of the device. This article when radiated with polychromatic light shows substantial changes in reflected wavelength with thickness changes of only a few Angstroms.

After affixing the anti-reflective layer or layers to the substrate, receptive material is attached to the top anti-reflective layer by various means. The top anti-reflective layer can be chemically activated to covalently bind the receptive material, or to adsorb the receptive material, or the receptive material can be coated onto the anti-reflective material by the Langmuir-Blodgett method. The function of the receptive material is to selectively adsorb, covalently bind or affix by any mechanism a specific analyte from any fluid containing the analyte, which is contacted with the coating.

After affixing the receptive material to the coated substrate, the assay apparatus has been produced. The precoated apparatus or article may then be utilized to assay any fluid suspected or believed to contain the analyte of interest. The fluid is exposed to the already coated or precoated device, and is allowed to incubate

for a predetermined period of time. Next the apparatus is rinsed to remove unbound material and dried. Positive results show a change in the visible interference color, negative results show no change in the interference color.

In the following examples, the top anti-reflective material onto which the receptive material is coated is a silicon oxide; however, any anti-reflective material capable of having receptive material adhered is a suitable substitute. Such examples of top anti-reflective layers include, but are not limited to, elemental carbon (in graphite-like or diamond-like lattice structures), boron compounds, organic polymers TiO_2 or alumina, silicide compounds or any other compounds that exhibit the ability to be activated to attach, adhere, affix or otherwise secure receptive material which is capable of selectively binding or interacting with the analyte in any sample fluid.

Subsequent qualitative or quantitative detection of the analyte is thereby made relatively easy since the reflection is diffuse and less angle dependent. Qualitative detection of the analyte is by the unaided eye, quantitative detection is performed with such instruments as the $Sagax_R$ comparison ellipsometer or a reflectometer, or any other instrument capable of measuring such physical parameter as wavelength, polarization, intensity, small density changes, etc.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 - Represents a prepared slide with an irregular surface, anti-reflective layers, receptive material.

Figure 2 - Represents a prepared slide reacted with analyte.

Figure 3 - Represents the diffuse or non-specular reflection of light from an uneven substrate.

Figure 4 - Represents the specular reflection of light from a planar substrate and the non-specular reflection of light from a planar substrate covered with plastic.

Figure 5 - Represents a prepared slide, coated with receptive material and partially covered with a non-specular material.

Figure 6 - Represents a prepared slide with an irregular surface and receptive material.

DETAILED DESCRIPTION OF THE INVENTION

The following definitions are supplied for the purpose of clarifying aspects of the invention and are not intended to limit the invention in any way:

Anti-Reflective Layers: A thin layer of material coated on a substrate to suppress unwanted reflections off the surface. The layer is most effective when the reflections from the outer surface of the layer and the outer surface of the substrate are approximately 180 degrees out of phase with each other.

Antibody: A class of serum proteins which specifically bind to an antigen which induced the formation of the antibody.

Antigen: Molecules which induce an immune reaction when recognized by the host's immune system.

Interference Phenomena: A destructive and/or constructive interaction of two or more lightwaves resulting in an intensity that has a different resulting sum than the sum of the original component waves.

Diffuse Reflection: Incident light reflected from a surface that has surface ridges and valleys which are large in comparison to the wavelengths of irradiated light.

Specular Reflection: Incident light reflected from a surface that has surface ridges and valleys which are small in comparison to the wavelengths of irradiated light.

Index of Refraction: Ratio of speed of visible light in a material to the speed of light in a vacuum.

Optical Thickness: The measurable physical thickness of a material multiplied by the index of refraction of the material.

Interference Color: The phenomena of one or more wavelengths of light constructively or destructively interfering, and thus suppressing certain wavelengths in a region of the spectrum and reflecting a color by an interference phenomenon.

What follows is a description of the invention including the steps of coating the substrate with anti-reflective material and receptive material to form the precoated device, the method of assaying for the analyte found in the fluid sample, and the method for detecting and determining the amount of analyte bound to the substrate without the use of markers or labels such as radioactive, enzyme, fluorescent, luminescent conjugates and the like. As such, the detection composition is unlabelled, comprising none of these markers. To clarify the following description, a specific example was selected, in which a silicon wafer served as the substrate, silicon monoxide and silicon dioxide were the coated anti-reflective layers and silane chemistry was utilized to bind bovine gamma globulin, the receptive material, to the apparatus. The method of preparing the apparatus is not limited to the specific materials selected in this example. Any compounds which fit the specific criteria listed for each of the elements of the apparatus can be utilized by slightly modifying the hereinafter described process.

STEP 1: Substrate Selection

This invention provides for diverse substrate formats; the substrate can be chosen to be a solid support, a flexible or semi-flexible support, a pellicle or a gel depending on the type of analyte chosen and the assay characteristics desired by the end user.

The present invention has three preferred embodiments: the first utilizes a substrate with an irregular surface to produce diffuse light reflection, the second utilizes a smooth substrate surface with a light diffusing or light modifying material such as smoked glass or textured plastic, etc. applied over the slide after the apparatus has reacted with the analyte, the third embodiment will be discussed later. The first and second embodiments of the present invention are prepared in a similar manner with the exception of preparation of the surface of the substrate. The first embodiment is formed with a substrate of suitable material with at least one irregular surface (which shall hereinafter be referred to as the irregular substrate). A simple method of generating an irregular surface is to use material with a lattice structure that forms an irregular pattern when cleaved. However, material with a lattice structure that forms a smooth surface when cleaved can also be utilized in the first embodiment (and obviously without further change in the second embodiment) of this invention. A smooth substrate surface can be roughened by etching (for example silicon in aqueous KOH) or by various other known processes to form a surface with irregular variations of height, sufficient to produce a diffuse reflection of light from the surface of the substrate.

Referring to Fig. 1, there is shown a substrate having a substantially irregular surface fabricated of any suitable material; in this example the selected substrate is a silicon wafer (commercially available from

Addison Engineering Inc.). The wafer is approximately 4 inches in diameter and .02 inches thick; it should be noted that these dimensions of the substrate are not critical to the present invention.

The substrate is formed from a silicon crystal which is diamond sawed to form a wafer which is then subjected to anisotropic etching in KOH, or isotropic etching to form a smoother surface. Then one surface of the wafer is polished producing a smooth mirror-like finish producing a specularly reflecting surface. The reverse surface remains slightly irregular with ridges and valleys on the order of 200-300 nm in height producing a diffusely reflecting surface. The variations in height of the surface imperfections can range from Angstroms to thousands of micrometers as long as a non-specular reflecting interference color change can be detected when the analyte binds to the receptive material. In accordance with the first embodiment of this invention the remaining steps are performed on the irregular substrate, in accordance with the second embodiment of this invention the smooth polished surface is utilized.

The third embodiment of this invention is prepared in a similar manner as either the first or the second embodiment with the exception of the affixation of the anti-reflective layers. The third embodiment is designed to be readable by an instrument such as an ellipsometer, photoreflectometer, comparison ellipsometer, etc., and not necessarily by the unaided eye; therefore the use of anti-reflective material which generates a visible interference color is optional in the third embodiment.

The instrumented device is formed by the same methods as the first and second embodiments with the exception that the substrate is not necessarily coated with anti-reflective material and the substrate can be

directly activated to allow the binding of receptive material.

It was a highly unexpected discovery that the device when produced without anti-reflective material generated a signal which was easily detected by a variety of optical instruments. It was also discovered that when the first and second embodiments were read under an instrument often filters were necessary to achieve the results generated by the device with no anti-reflective material coated on the substrate (see Figure 5 and 6).

STEP 2: Affixation of the Anti-reflective Layers onto the Substrate

Silicon monoxide an anti-reflective (AR) thin film is coated on to the substrate by standard thin film coating techniques known in the semiconductor and optical industries. The coating can be performed by electron bombardment or by evaporating the material on to the apparatus in a low temperature vacuum chamber, capable of producing a vacuum of at least 10^{-5} torr or by any other equivalent method. These silicon based coatings are commercially coated, to the hereinafter mentioned specifications.

The material was coated to a thickness between 450 Angstroms and 650 Angstroms which is slightly less than a quarter of a wavelength optical thickness of visible light. Alternatively, the anti-reflective material can be coated between 1800 Angstroms and 12,500 Angstroms, which is approximately the thickness of three quarters to several quarters wavelength optical thickness of visible light to produce a more sensitive apparatus. The selected thickness of the anti-reflective coating is subject to wide variation and need not be a quarter wavelength optical thickness of AR material to be within the scope of this invention; although the preferred

thickness of a single AR coating is an odd number of one-fourth wavelengths optical thickness.

On top of the first anti-reflective layer is a second anti-reflective layer of silicon dioxide which is coated to a thickness of approximately 50 Angstroms. This layer is coated in a similar manner as the first layer.

Whenever incident light passes from one medium into a second medium with a different refractive index, a portion of the light is reflected at the interface and a second portion of the light is transmitted. The desired ratio of reflected/transmitted light can be achieved by coating thin layers of anti-reflective material. The anti-reflective layers coated onto a substrate act to cancel, by destructive interference, the reflections from the outer surface of the layer and the outer surface of the substrate. To achieve partial destructive interference the reflections must be of equal intensity and approximately 180 degrees out of phase. When the AR coatings are coated with a thickness of lambda/4 and lambda is the chosen wavelength, then the reflections will be out of phase.

To achieve a minimum reflectance of incident light lambda/4 should be approximately equivalent to the optical thickness of the coated material. Optical thickness is defined as the physical thickness of the coating multiplied by the refractive index of the AR materials. Therefore, in the preferred embodiment of this invention the optical coatings are usually deposited with a thickness of 1/4 wavelength optical thickness.

However, it is necessary not only to coat the correct thickness; it is also necessary to have reflections of equal intensity. To establish equal intensities the refractive indices should form a geometric progression. Since the refractive index of air is 1.0 the refractive index of the AR coating should be

approximately equivalent to the square root of the substrate. In other words the greatest reduction in reflection occurs for an anti-reflective coating having a thickness of approximately one-fourth wavelength optical thickness of light in the medium, and a refractive index which is the square root of the product of the indices of the media directly above and below it. This thin film of silicon monoxide provides the apparatus with a coating having a refractive index of 1.8-2.0 which is approximately the square root of the refractive index of the silicon wafer which is 4.1. Thus affixation of anti-reflective layers diminishes the loss of incident light due to reflection.

The number of anti-reflective layers coated onto the substrate can be any integer 1 to infinity with the preferred number of layers of material being two. The number of layers is limited only by two factors: the indices of refraction of the selected materials should follow a geometric progression, and the cost involved in multilayer coating.

The slide apparatus when coated with the silicon anti-reflective layers suppresses certain wavelengths in the visible blue light range and therefore reflects a gold interference color. Although a gold interference color is utilized in this combination of elements the visual interference color of the slide can be any suitable color in the spectrum; depending on the material of the substrate selected, the chemical composition of the anti-reflective layers selected, and the thickness and number of the layers coated.

STEP 3: Adhering Receptive Material to the Slide Apparatus

Prior to adhering the receptive material to the optically coated substrate, the surface of the apparatus can be thoroughly stripped of foreign particles, which

could cause non-specific binding, or background signals. To accomplish this the apparatus is oxygen plasma etched in a vacuum chamber, containing oxygen at a partial pressure through which an electrical charge is applied. The coated apparatus is placed into a vacuum chamber, which is excavated to 0.7 Torr. The oxygen is excited by a plate current of 175 D.C. millamperes, 250 RF watts to form an oxygen plasma.

The oxygen plasma etching process is allowed to continue for five minutes to assure the removal of all undesired organic matter. This oxygen plasma etching process should be performed immediately prior to adhering the receptive material.

The cleaned apparatus then is chemically activated to facilitate the covalent binding of the receptive material to the top coated anti-reflective material, silicon dioxide. Adherence of the receptive material can be done by alternative methods such as Langmuir-Blodgett coating techniques or by adsorption or by any other mechanism which will adequately affix the receptive material to the activated coated substrate. As previously discussed in the third embodiment the cleaned substrate is chemically activated to facilitate the adherence of the receptive material to the device.

Because this example is coated with silicon dioxide, silane chemistry is used to activate the surface and covalently bind the receptive material to the surface. Twenty five coated wafers are placed into a quartz rack which is inserted into a vacuum dessicator; within this dessicator a small vessel is inserted containing approximately 5 microliters of bisamino-silane. Specifically, N-(2-aminoethyl)-3-aminopropyl-trimethoxysilane having a boiling point of 140 degrees at 15 Torr is used. The boiling point of the aminosilane compound determines the optimal pressure and temperature for running the vapor phase deposition.

The vacuum dessicator is evacuted to 0.06 Torr for 30 minutes. Then the temperature of the dessicator is raised to 100 degrees C over the course of one hour, to produce a coating of active material of approximately 20-30 Angstroms. This coating remains stable for at least 6 months.

The chemical activation coating can be performed by alternative methods as depicted in the examples. Other methods include, but are not limited to, chemical activation by solution deposition, by spin coating, or by any other mechanism capable of preparing the surface for affixing the receptive material(s) to the surface of the apparatus.

The apparatus is now prepared for the attachment of the receptive material. This specific test is for the detection of rheumatoid factor, the analyte, in a body fluid. Three acceptable receptive materials or ligands, namely Bovine Gamma Globulin, human IgG, or Bovine Immunoglobulin G (IgG), have been utilized to bind the analyte. For differing analytes different receptive material can be utilized. This example uses the most cost efficient ligand Bovine Gamma Globulin (hereinafter called BGG). The coated apparatus is placed into a 8 cm by 3 cm by 2 cm plastic cell culture box (any suitable container can be used). Twenty mls of 10 mM phosphate buffered .9% saline solution (hereinafter referred to as PBS) adjusted to a pH of 7.4 is combined with 200 micrograms per ml of BGG (the ratio of weight/volume gives an excess of ligand and it is subject to a wide range of dilution), and placed into the plastic box. To this solution is added a ratio of 1% per volume of glutaraldehyde. The solutions in which the receptive material is mixed will depend on the characteristics of the activation step and on the choice of receptive material.

The box containing the apparatus that is covered by the receptive material solution is incubated at room temperature in an agitation bath. The agitation enhances the uniform adherence of the receptive material to the coated apparatus. The binding of the BGG to the apparatus in this example is by the formation of covalent bonds between the activated silane functional groups and the protein molecule. The adherence of the chosen receptive material can be performed by a variety of known techniques. The preferred technique will be a function of the type of binding required and the type of receptive material employed.

Following incubation, unbound bovine gamma globulin proteins are removed from the apparatus by rinsing the apparatus thoroughly with distilled deionized water. The preparation of the pre-formed article is completed by placing the apparatus under a stream of N_2 or pressured air, or by air drying the apparatus (see Figure 1). However, in the preferred embodiment of this invention an optional blocking step is implemented prior to drying the slide.

Following the rinsing of the slide apparatus to decrease the amount of non-specific binding the apparatus is placed in a blocking solution and allowed to incubate for one hour in an agitated bath. The solution was made of 2 ug/ml of acid hydrolyzed casein plus a ratio of 1% glycerol volume to volume and 2% sucrose weight to volume in PBS sufficient to bring the total volume to 20 mls. Again, the slide must be covered by solution and if needed more PBS can be added to the solution.

Following the agitated incubation the apparatus is rinsed with deionized distilled water to remove all unbound blocker, and the slide is dried as previously described. Various blocking agents and blocking solutions known to those skilled in the art can be

utilized for example BSA, milk, spermidine, glycine, ethylene diamine, etc.

STEP 4: Assay for Analyte Affixed to the Apparatus

Prior to contacting the fluid containing the analyte with the pre-formed article, the fluid is diluted by adding aliquots of the fluid to phosphate buffered saline. Although dilution is not necessary it is the preferred means for lessening non-specific binding. For this example, the optimum preferred dilution for this assay is 1:1 (volume serum:volume diluent), although wide latitudes of dilution can be used, depending upon the nature of the fluid to be assayed and the assay detection technique employed. Alternative sample diluents can be utilized to prevent adventitious adherence of undesired material to the pre-formed article, for example 20 mM TRIS at a pH of 8.0.

The diluted sample of fluid suspected of containing the analyte of interest is then placed into contact with the pre-formed slide. The pre-formed article can be dipped into the diluted sample, or a small drop of the fluid can be smeared or lightly contacted with the surface of the article. Adherence of the analyte to the article is enhanced by incubation of the slide on a heating block at 45 degrees C for three minutes, or optionally the pre-formed article can be placed under a heat lamp for five minutes, or alternatively, the device can incubate at room temperature until the moisture in the sample has evaporated. It should be understood that the temperature at which the device incubates is a function of the receptive material and analyte selected.

Following the incubation, the pre-formed article is fully rinsed to remove from its surface any material i.e. any unbound solution containing analyte which has not adhered to the coated surface. Then the device is

dried with a stream of N_2 or pressurized air, or any equivalent means for removing the rinsing solution and preventing water spotting on the slide's surface (see Figure 2).

STEP 5: Assay Detection Techniques

Two assay detection techniques, visual and instrumental, are primarily used for the detection of the bound analyte, although any suitable means of detection can be employed. For the examples described hereinafter, an interference color phenomena is used to visually ascertain whether the fluid contains the analyte of interest.

The pre-formed article can be irradiated with a polychromatic or monochromatic light source to determine whether analyte has bound to the surface. The pre-formed (or unreacted) article under polychromatic light reflects a gold interference color with an absorbance maxima of approximately 476 nanometers, in comparison the reacted article under polychromatic light reflects the gold color where no analyte is bound; and it reflects a purple or a blue interference color with an absorbance maxima between 550 nanometers and 650 nanometers where the analyte is bound. The color of the reacted spot depends on the concentration of the analyte bound to the surface. The higher the concentration the more intense the color change appears.

When the analyte binds to the receptive material the physical thickness of the material on top of the substrate either increases or decreases. This thickness change results in a change in the optical pathway; the new optical pathway causes the interference color to change. In order to achieve the correct phase shift for destructive interference in the preferred method of this invention, the thickness of the SiO, or of any AR film coating, is slightly less than an odd number of quarter

wavelengths of optical thickness. Because air is a less dense medium than the AR coats or the substrate there is a pi/2 phase shift for reflections at both interfaces. The identical phase shift cancels each other.

Therefore, to determine the net phase shift the only factor is the optical path difference, which is defined as $2d \times n_f$. The definition of d is the actual measurement of the AR thickness, and n_f is defined as the AR film's refractive index. The actual phase shift is therefore 2dn/lambda multiplied by 2pi if in radians, or 360 if in degrees. The thickness of the AR is some odd quarter wavelength optical thickness; in this example the actual optical thickness is approximately equivalent to lambda/4, lambda being the wavelength selected for peak performance. Therefore, the phase shift is about 180 degrees and there is partial destructive interference which reveals a gold interference color because the certain wavelengths in the blue region at approximately 465-480 nanometers are suppressed.

When the analyte binds to the receptive material on top of the article, the optical path is either increased or decreased. In this example, the actual thickness change incurred by the binding reaction between the rheumatoid factor and the Bovine Gamma Globulin is an increase in material of approximately 20 Angstroms. This change in the optical path yields a suppression of the gold wavelengths on the order of 450 nanometers, thus resulting in a blue or purple interference color defining the area of binding of the analyte. The purple color on the irregular surfaced substrate is diffusely reflected (see Figure 3), the purple color on the smooth surfaced substrate is specularly reflected prior to addition of the light diffusing material (see Figure 4).

To avoid the need for signal enhancement techniques the interference color change should be optimized to yield a contrasting interference color when

reacted. This provides a color contrast to which the human eye is highly sensitive. It should be understood that any color shift whether visible to the human eye or not can be utilized as a detection signal, because the instrumented detection method is highly sensitive to nonvisible changes in thickness. The instrument frequently used to quantitatively analyze the amount of bound material is the ellipsometer, although various other instruments can perform a similar analysis. Although all three embodiments of the present invention can be read by an instrument, the third embodiment is specifically designed for instrumented detection. The instrumented detection allows the assay to be performed on a specularly or non-specularly reflecting surface with comparative results.

Ellipsometry is an optical method for determining the thickness and refractive index of extremely thin films. The technique exploits changes from planar to elliptical polarization which occur as the light beam is reflected. The shape and orientation of the ellipse are affected by the angle of incidence and properties of the reflective surface. Elliptical orientation is thus a very sensitive measure of the thickness of a film coating the reflective surface. The proper incidence angle for a comparison ellipsometer yields optimal detection of film thickness to about 3 Angstroms. This corresponds to a sensitivity of approximately 1/1000 the wavelength of the light illuminating the surface.

The Sagax$_R$ comparison ellipsometer compares the thickness of the reacted slide with a reference film that has a thickness nearly equivalent to the unreacted slide. The polarized light is reflected off both surfaces and converted into a digitized image. The image is converted into gray scale values in accordance with the light

intensity. These gray scale values are a faithful representation of the actual film thickness.

Known concentrations of analyte reacted on an article were read on the ellipsometer and the resultant data was used to generate a standard curve. The concentration of the analyte, rheumatoid factor, in this sample is calculated in relation to the previously generated standardized curve. It should be apparent that other methods of measurement of the analyte concentration can be employed within the scope of the present invention.

In the second embodiment of this invention, the regular surfaced substrate was used. To produce the less angle dependent diffuse reflection the article is covered with a light diffusing material prior to the visual or instrumented detection of the analyte. This material can be permanently coated or affixed onto the article or alternatively it can be removable. An opaque non-glare piece of glass approximately four inches in diameter and 1/16th of an inch in thickness was placed over the second embodiment to produce a diffuse reflection or an attenuated reflection. Materials such as opaque plastics, smoked glass, or any other functional equivalent material, can be used to produce a diffuse reflection an attenuated reflection, or a light scattering effect.

EXAMPLE 1

Detection of Rheumatoid Factor Using Thick Anti-Reflective Coatings

A silicon wafer approximately four inches in diameter and .02 inches in thickness having a highly polished surface and an irregular surface was coated on both sides with a combination of silicon monoxide and silicon dioxide to a thickness of 11,893 Angstroms of anti-reflective material. This wafer was cut in half and

one sample assay was performed on the irregular surface, the second sample assay was performed on the smooth surface. This coated wafer was activated by application of N-(2-aminoethyl)-3-aminopropyl trimethoxysilane by the following procedure:

1. The coated wafer was oxygen plasma etched for five minutes in a vacuum at 0.7 Torr, with an oxygen atmosphere and a plate current of 175 D.C. milliamperes and 250 RF watts.

2. Immediately upon removal the coated wafers were placed in a quartz rack and inserted into a vacuum dessicator with a vessel containing 5 microliters of N-(2-aminoethyl)-3-aminopropyltrimethoxysilane. The vacuum was run at 0.06 Torr for 30 minutes. Then the temperature of the dessicator was raised to 100 degrees over the course of one hour to complete the vapor phase deposition of aminosilane.

3. 200 micrograms/per ml of BGG and 20 mls of PBS (previously described) and 1% by volume of glutaraldehyde were combined to form the receptive material solution. The wafers were placed in a petri dish and the receptive material solution was added.

4. To enhance the adherence of the receptive material the wafers were allowed to incubate at room temperature in an agitation bath for 15 hours.

5. Following incubation the unbound bovine gamma globulin proteins were removed from the wafers, by rinsing with distilled deionized water.

6. Following the rinsing step the wafers were placed in the previously described casein blocking solution to decrease non-specific binding. The petri dish containing the wafers and the blocking solution were incubated at room temperature in an agitating bath for one hour.

7. Following the blocking process all excess blocker was removed by rinsing with deionized distilled water. Then the slide was dried under a stream of nitrogen. The pre-formed wafers were then used to determine the presence of rheumatoid factor in two positive serum samples. A smooth surface assay and an irregular surface assay as described hereinafter, using the coated wafer prepared as previously described, generated the data in Table 1 by the following procedures:
   a. Each serum sample was diluted by adding PBS (previously described) at a 1:1 ratio (volume serum: volume diluent).
   b. 5 microliters of each diluted sample was placed on each wafer; and the wafers were allowed to incubate on a heating block at 45 degrees C for three minutes to enhance analyte adherence.
   c. The wafers were then rinsed with deionized water to remove all unbound material, then dried with a stream of pressurized air.

Upon visual inspection of the unreacted portion of the irregular wafer under polychromatic light, the wafer reflected a diffuse green color at most viewing angles. Although at more oblique angles a diffuse rose color appeared. The two positive sera samples reflected a diffuse rose colored spot which was visible at most viewing angles, but shifted to a green colored spot at more oblique angles. When the diffusely reflecting wafer

was placed under a green monochromatic light with a wavelength of 546 nanometers the unreacted surface diffusely reflected a green color and the reacted portion reflected a black spot at most viewing angles. Similar inspection of the unreacted, specularly reflecting wafer under polychromatic light revealed a green specular reflection at normal to 30 degrees off normal viewing angles and at angles greater than 30 degrees the wafer appeared rose colored. Dependent on the angle of incident light the reacted spot was visible. The smooth wafer was then covered by a light diffusing plastic sheet approximately 0.01 inches in thickness which made the reacted spot more easily distinguishable from the background color. The plastic also allowed the spots to be visible at angles of incident light greater than 30 degrees from normal.

TABLE 1

Thickness Change of Reacted Wafers
Due to the Binding of Rheumatoid Factor

Diffusely Reflecting Wafer	Reacted Wafer Thickness	Wafer Thickness	Thickness Change
Positive Sample 1	12,003 A	11,893 A	110 A
Positive Sample 2	12,032 A	11,893 A	139 A
Specularly Reflecting Wafer Covered With Light Diffusing Material	**Reacted Wafer Thickness**	**Wafer Thickness**	**Thickness Change**
Positive Sample 1	11,999 A	11,890 A	109 A
Positive Sample 2	12,031 A	11,890 A	141 A

Thickness changes measured in Angstroms.

EXAMPLE 2

Detection of IgE Antibodies Specific for Birch Pollen

A silicon wafer four inches in diameter and .02 inches in thickness, with a smooth surface and an irregular surface was coated on both sides with two anti-reflective layers, one of silicon monoxide at approximately 500 Angstroms thick and a second of silicon dioxide to a thickness of 50 Angstroms. These coatings were done by an outside company. The irregular coated surface of the wafer measured 531 Angstroms prior to the activation step. The wafer was activated by application of diluted purified nitrocellulose by the following procedure:

1. The wafer was placed on a photoresist spin coater and washed with 3 mls of acetone to remove foreign particles.

2. 300 microliters of the hereinafter described solution was then sprayed onto the spinning wafer. The solution consisted of 0.0829 grams of purified nitrocellulose (available from Balzer's Union) dissolved in four mls of pentyl acetate.

3. The activated wafer was then removed from the spin coater and heated in an oven at 120 degrees for one hour. The heating process aided in eliminating any excess pentyl acetate, and aided the adsorption of the nitrocellulose to the anti-reflective material. The activation material was measured at 552 Angstroms on an ellipsometer.

4. The receptive material, birch pollen extract, was made by dissolving 1/2 gram of birch pollen (commercially available) into 5 mls of a 10% isotonic phosphate buffer comprised of 2.2 grams of dibasic

potassium phosphate with 0.1% Tween by volume in 1000 mls of distilled water adjusted to a 7.5 pH.

5. The birch pollen buffered solution was stirred for 3 hours to extract the surface allergens from the birch pollen. After being stirred the solution was centrifuged for 20 minutes at 1500 RPM, after the centrifuge process the supernate liquid containing the birch pollen extract was decanted off and stored. The centrifuge process was then repeated and again the decanted liquid was kept.

6. The 1/2 ml of the birch pollen extract was diluted in 20 mls of PBS and placed on top of the wafer in a petri dish. The petri dish was then agitated overnight at room temperature to facilitate the adsorption of the receptive material to the nitrocellulose activation material. The blue colored wafer was measured and 39 Angstrom increase was recorded.

The pre-formed wafer was then used to determine the presence of IgE antibodies in four serum samples from individuals with:

1. Acute allergic response (Acute)
2. Strong allergic response (Strong)
3. Mild allergic response (Mild)
4. No apparent response (Normal)

An assay as described hereinafter, using the coated wafer, prepared as previously described, generated the data in Table 2 by the following procedures:

1. 5 microliters of each serum sample were placed on the surface of the wafer. The wafer was then incubated at 37 degrees C for 15 minutes to evaporate all moisture from the sample, and to facilitate the

adherence of the IgE antibodies specific for birch pollen.

2. The wafer was rinsed briefly in deionized distilled water to remove all unbound material. Then the wafer was placed under a stream of N_2 to dry the surface of the wafer. Upon visual inspection of the wafer under polychromatic light, three of the serum samples showed diffusely reflecting white spots against the bright blue background, the normal sample showed no visual color change. The intensity of the white spot correlated well with the level of allergic response. The acute sample was an extremely bright spot, the high sample was slightly less distinctive and the mild spot was readily visible but less distinctive than the other two samples.

3. The change in the thickness of the reacted portion of the wafer was measured in Angstroms on an ellipsometer.

TABLE 2

Thickness Change due to IgE Antibody Adherence

	Concentration of IgE in Sera	Total Angstrom Thickness	Increase Due To Analyte
Acute	3.5 nm/ml	1230 A	109 A
Strong	.7 nm/ml	1179 A	58 A
Mild	.35 nm/ml	1153 A	32 A
Normal	0 nm/ml	1135 A	14 A*

*Due to non-specific binding.

EXAMPLE 3

Detection of Carcinoembryonic Antigen (CEA)

A silicon wafer with a highly polished side and an irregular rough side was coated on both sides with an anti-reflective material, silicon oxynitride, to a thickness of between 500 -650 Angstroms of material by Meadowlark Optics. The irregular side surface was chemically activated by application of N-(2-aminoethyl)-3-aminopropyl trimethoxysilane by the following procedure:

1. The coated wafer was oxygen plasma etched for 5 minutes in a .71 Torr oxygen atmosphere with a plate current of 175 D.C. milliamperes and 250 RF watts.

2. Immediately upon removal from the oxygen plasma etcher the wafer was placed in a quartz rack which was inserted into a vacuum dessicator with an attached vessel into which 5 microliters of the aminosilane was placed.

3. The vacuum dessicator was run at .06 Torr for 30 minutes. Then the temperature of the dessicator was raised to 100 degrees over the course of one hour. This process activated the wafer by the vapor deposition of the aminosilane onto the water surface.

4. The wafer was now prepared for the adherence of the receptive materials. First, a solution comprised of 20 ml PBS, .1% glutaraldehyde by volume, and 150 microliters of IGAP which is a synthetic polypeptide that covers the active region of protein A was made up. (Other experiments substituted protein A or protein G for the IGAP and similar results were achieved.)

5. 200 microliters of monoclonal antibody (polyclonal can be substituted) specific for carcinoembryonic antigen was pipetted into the IGAP solution at 1 mg/per ml.

6. The combined solution was placed on top of the wafer in a petri dish and was incubated at room temperature in a shaker bath for two hours. When removed the petri dish was transferred to a cooling unit and allowed to incubate for 48 hours at 4 degrees C to further the adherence of the receptive antibody material. To remove all unbound material the wafer was rinsed with distilled deionized water and dried in a stream of N_2.

The pre-formed wafer which diffusely reflected a tan color was then used to determine the presence of CEA antigen in five serum samples with known CEA concentrations.

An assay as described hereinafter, using the coated wafer, prepared as just described, yielded the data in Table 3 and the data in Chart 1 by the following procedures:

1. 10 microliters of all five samples of serum were contacted with the surface of the wafer, and allowed to incubate at 37 degrees C for seven minutes.

2. After seven minutes the wafer was rinsed in deionized distilled water to remove all unbound material. Then the wafer was dried under a stream of nitrogen. Visual inspection of the slide revealed small pinkish spots with color

intensities which correlated with the increasing analyte concentration levels.

3. The change in the thickness of the reacted portion of the wafer was measured in gray scale units by a Sagax$_R$ comparison ellipsometer.

TABLE 3

Measurement of Bound CEA Antigen

CONCENTRATION (ng/ml)	Average Gray Scale	Standard Deviation	CV	+/- 2 SD
0.0	41.0	2.68	6.6%	35.6-46.4
0.5	48.3	2.95	6.1%	42.4-54.2
0.9	51.7	2.59	5.0%	46.5-56.9
3.7	65.1	7.02	10.8%	51.1-79.1
7.5	98.8	3.79	3.8%	91.2-106.4

EXAMPLE 4

Detection of Group A Streptococcus

Three silicon wafers with highly polished surfaces, and three silicon wafers with non-regular surfaces each having a refractive index of approximately 4 to 4.08 at a specified wavelength, were used in this test. Group A was coated with approximately 550 Angstroms of silicon monoxide having an index of refraction of 1.97 at a specified wavelength. Group B was coated with approximately 560 Angstroms of silicon oxynitride having an index of refraction at a specified wavelength of 1.95. Group C was coated with approximately 550 Angstroms of boron oxide having an index of refraction at a specified wavelength of 1.9. These coatings closely approximate the equation used for coating one optical layer to the surface of the substrate.

The receptive material was coated to a thickness of 25 to 30 Angstroms. This figure was disregarded in calculating the optimal thickness of the anti-reflective material. The three groups of wafers were chemically activated to allow attachment of the receptive material. The activation was by application of N-2(2-aminoethyl)-3-aminopropyltrimethoxysilane by the following procedure:

1. The coated wafers were oxygen plasma etched for 5 minutes in a 0.70 Torr oxygen atmosphere with a plate current of 175 D.C. milliamperes and 250 RF watts.

2. Immediately upon removal from the oxygen plasma etcher the wafers were placed in a quartz rack which was inserted into a vacuum dessicator with an attached vessel into which 2.5 microliters of the aminosilane was placed.

3. The vacuum dessicator was evacuated to 0.06 Torr for 30 minutes. Then the temperature of the dessicator was raised to 100 degrees C over the course of one hour. This process activated the wafer by the vapor deposition of aminosilane onto the wafers' surfaces.

4. The wafers were now prepared for the adherence of the receptive materials. First, 250 micrograms per milliliter of Bovine Serum Albumin (BSA) was dissolved in Phosphate Buffered Saline (PBS). 10 milliliters of this solution was adjusted to pH 8.5 and was pipetted into a plastic culture cell box containing the six wafers.

5. 50 microliters of a 25% glutaraldehyde in water solution was added to the culture cell box. 25 microliters of a 50 ml micrograms per ml of a Protein A solution was also added to the culture cell box.

6. The six wafers in the culture cell box were incubated in a shaker bath at room temperature for one and a half hours.

7. 200 microliters of a commercially available (from Ventrex, Inc.) anti-Strep A (raised in rabbits) was pipetted into the culture cell box; and allowed to incubate for another hour in the shaker bath at room temperature.

8. Following incubation the culture cell box was refrigerated at 4 degrees C for approximately 48 hours to further adherence of the receptive antibody material to the device. To remove all unbound material the wafers were rinsed with distilled deionized water and dried in a stream of N_2.

The six wafers appeared shades of gold with three of the wafers providing a specular reflection and three of the wafers providing a non-specular reflection. These wafers were then used to determine the presence of Strep A in either a bacteria form or a bacteria lysate form.

An assay technique is described hereinafter:

1. Each wafer was separated and placed into a small plastic test kit. The test kit was designed with two rough grade filter paper pads attached to the inside of the top cover. Each test kit has seven microliters of a positive control placed on one pad and seven microliters of a negative control placed on the second pad. The positive control, commercially obtained from Becton Dickinson, was heat killed Group A streptococcus in solution with a .02% sodium azide. The negative control was taken from a solution of 50 micrograms of BSA per ml of PBS.

2. The cover of the test kits were closed placing the filter pads in contact with the coated wafer for one minute.

3. The covers were opened and the wafers were allowed to air dry for one minute. Then the wafers were rinsed with distilled deionized water and dried with pressurized air.

Visual inspection of the wafers revealed highly visible purple spots on the non-specular wafers and visible purple spots on the specular wafers. Visual inspection of the non-specular wafers demonstrated a lower angle dependence than that of the specular wafers. The reacted colors became less vibrant at lower concentrations, although all spots were clearly distinguishable. An instrument was used to verify the increase of mass produced by the binding reaction on the surface of the wafer. The change in mass or thickness was measured in gray scale units by a Sagax$_R$ Comparison Ellipsometer.

The foregoing examples serve to illustrate the efficiency and utility of this technology to detect a variety of analytes using the pre-formed slide consisting of a substrate, anti-reflective material(s), activation, and receptive material(s) to generate an interference color change as a signal of analyte attachment.

Without being bound to the substrate formats or materials utilized in the preceding examples, it is possible to utilize a diversity of combinations of substrate formats and substrate materials which are functionally equivalent substitutes capable of having anti-reflective material bound to their surface, or as described in the third embodiment are capable of being activated to allow attachment of the receptive material (see Figure 5).

The anti-reflective material(s) provides a layer which can be activated to function as the receptive material or to attach or adhere, by whatever mechanism the receptive material to the apparatus. Furthermore, the anti-reflective material utilized in the preferred embodiment of this invention is coated to be a quarter wavelength optical thickness of a designated wavelength of light to achieve the highest level of destructive light interference. The AR coating can vary from a quarter of the designated wavelength namely, because destructive interference need not be total to achieve an adequate interference color. The actual amount of AR material coated to the surface can vary widely because the designated wavelength can be almost any wavelength.

The material composition of the AR layers is selected based on the refractive index of the AR materials and the substrate materials selected; therefore almost any AR material can be utilized if the correct combination of materials is selected.

The top AR material is selected to be capable of being activated to function as, or to receive, the receptive material. Most AR materials can be activated by some means to allow attachment of receptive material to the apparatus; therefore a multitude of materials is capable of being utilized as anti-reflective materials.

Various methods of chemical activation can be utilized dependent on the composition of the AR material and the receptive material, or if the activation material(s) are acting as the receptive material then dependent on the composition of the AR material(s) and the analyte. A variety of materials, functional groups, etc. can function as the activation material or as the activation process.

The treatment of the substrate, which can be any of a variety of shapes, i.e. test tubes, wafers, glass slides, microwells, etc., and formats, i.e. a solid

support, a flexible support, a gel, a pellicle and made of various suitable materials i.e., glass, silicon, plastics, such as polystyrene and the like and/or comprises a coating thereof on another support material etc., with the aforementioned technology affords many useful approaches to the detection of the analyte. This analyte detection need not be limited to visual detection of an interference color change. The color change can be in the infrared or the ultra-violet regions wherein the analyte detection is pursuant to an instrumented detection apparatus. The reacted apparatus can be qualitatively or quantitatively analyzed by an ellipsometer, or any other instrument capable of detecting by whatever mechanism the analyte binding.

The diffusely reflecting surface can be produced by using an irregular surfaced substrate, or by placing a suitable light diffusing or light interfering component or material over the top of the apparatus so that at least some of the layer capable of emitting a non-specular reflection is impinged upon, or by coating the substrate with material capable of producing a diffusely reflecting surface, or by any other suitable means for producing a diffuse reflection, an attenuated or less than total reflection, or any other non-specific reflection.

It is contemplated that the inventive concepts herein described may have differing embodiments and it is intended that the appended claims be construed to include all such alternative embodiments of the invention except insofar as they are limited by the prior art.

CLAIMS

What is claimed is:

1. An unlabelled article producing a non-specular reflection comprising a substrate capable of supporting on its surface not less than one layer of anti-reflective material adhering thereto and capable of being activated to attach analyte receptive material;

said analyte receptive material capable of selectively interacting with the analyte of interest, whereby a signal is generated.

2. An article as in claim 1 where the non-specular reflection is effected by employing a textured surface light transmissive material over some portion of said article.

3. An article as in claim 1 where the non-specular reflection is effected by employing a light modifying component extending over some portion of said article.

4. An article as in claim 1 where the non-specular reflection is effected by employing an irregular substrate.

5. An article as in claim 1 where the non-specular reflection is effected by employing an irregularly coated substrate.

6. An article as in claim 1 wherein the substrate has an irregular surface.

7. An article as in claim 1 wherein the substrate is a solid support.

8. An article as in claim 1 wherein the substrate is a flexible support.

9. An article as in claim 1 wherein said substrate is a pellicle.

10. An article as in claim 1 wherein said substrate is a gel.

11. An article as in claim 1 wherein said substrate is a plastic.

12. An article as in claim 1 wherein said substrate is a glass.

13. An article as in claim 1 wherein said substrate is a metal.

14. An article as in claim 1 wherein said substrate is a non-metal.

15. An article as in claim 1 wherein said substrate is supported by a support surface.

16. An article as in claim 1 wherein said substrate is light reflective.

17. An article as in claim 1 wherein said substrate is light transmissive.

18. An article as in claim 1 wherein the presence of the analyte is visually enhanced by utilizing polarizers.

19. An article as in claim 1 wherein the presence of the analyte is visually enhanced by irradiating the coated substrate with monochromatic light.

20. An article as in claim 1 wherein the presence of the analyte is visually enhanced by irradiating the coated substrate with polychromatic light.

21. An article as in claim 1 wherein the presence of the analyte is visually enhanced by irradiating the coated substrate with ultraviolet light.

22. An article as in claim 1 wherein the presence of the analyte is visually enhanced by irradiating the coated substrate with infrared light.

23. An article as in claim 1 wherein the analyte detected is evidence of Rheumatoid factor.

24. An article as in claim 1 wherein the analyte detected is IgE antibodies specific for Birch Pollen.

25. An article as in claim 1 wherein the analyte detected is carcinoembryonic antigen.

26. An article as in claim 1 wherein the analyte detected is streptococcus A.

27. An article as in claim 1 wherein the analyte detected is evidence of a viral infectious disease.

28. An article as in claim 1 wherein the analyte detected is evidence of an autoimmune disease.

29. An article as in claim 1 wherein the analyte detected is evidence of an allergen.

30. An article as in claim 1 wherein the analyte detected is evidence of a tumor marker.

31. An article as in claim 1 wherein the analyte detected is evidence of an infectious microorganism.

32. An article of claim 1 comprising not less than one discrete layer of anti-reflective material supporting thereon one discrete layer of activatable analyte-receiving material.

33. An article as in claim 1 wherein said anti-reflective material layer has a thickness of at least one quarter of a wavelength optical thickness of visible medium light.

34. A method assay which comprises the steps of:

contacting an already coated substrate having a top layer of material activated to allow attachment of a receptive material, with a solution believed to contain an analyte of interest;

facilitating interaction of the analyte to said receptive material; and

obviating the use of markers in detecting the presence of said analyte in a non-specular reflection.

35. A non-specular reflecting article comprising a substrate capable of activation allowing attachment of receptive material, said substrate supporting a layer of said receptive material capable of interacting with an analyte and obviates the use of markers to generate a signal.

36. An article defined in claim 35 having a substrate with an irregular surface.

37. A process of performing an instrumented assay which comprises contacting an article having a substrate capable of activation;

contacting said article with and allowing attachment of a receiving material;

contacting said article with a solution believed to contain an analyte of interest, facilitating adherence of the analyte to said receiving material and detecting by instrumentation the presence of said analyte in a non-specular reflection.

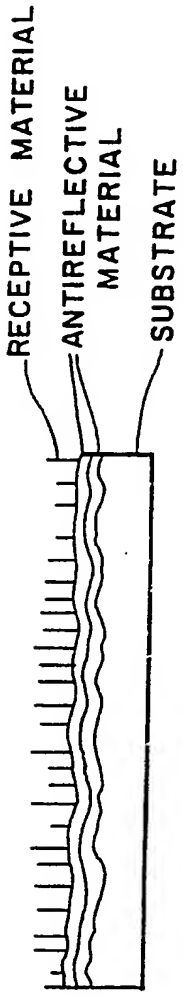
RECEPTIVE MATERIAL
ANTIREFLECTIVE MATERIAL
SUBSTRATE

FIG. 1.

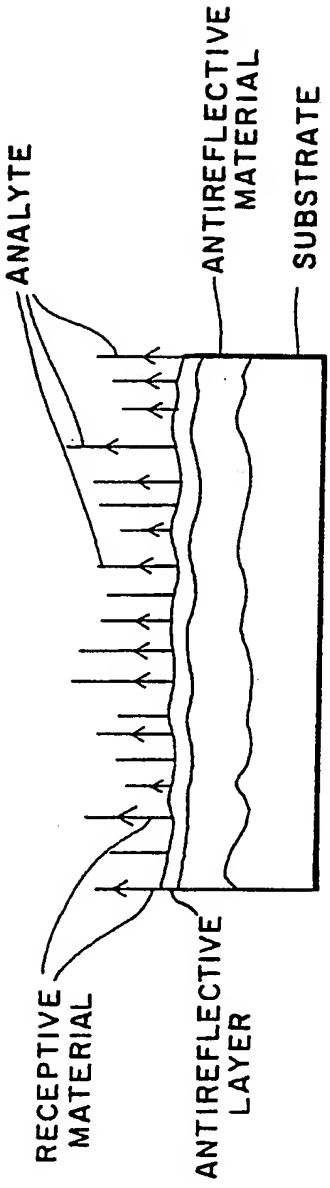
ANALYTE
ANTIREFLECTIVE MATERIAL
SUBSTRATE
RECEPTIVE MATERIAL
ANTIREFLECTIVE LAYER

FIG. 2.

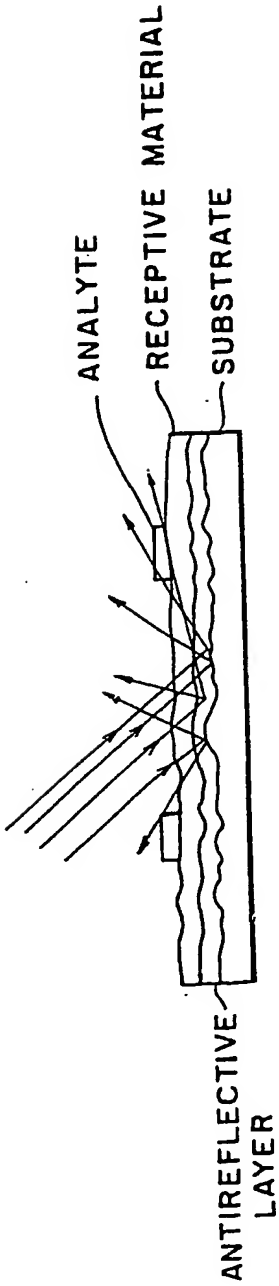
ANALYTE
RECEPTIVE MATERIAL
SUBSTRATE
ANTIREFLECTIVE LAYER

FIG. 3.

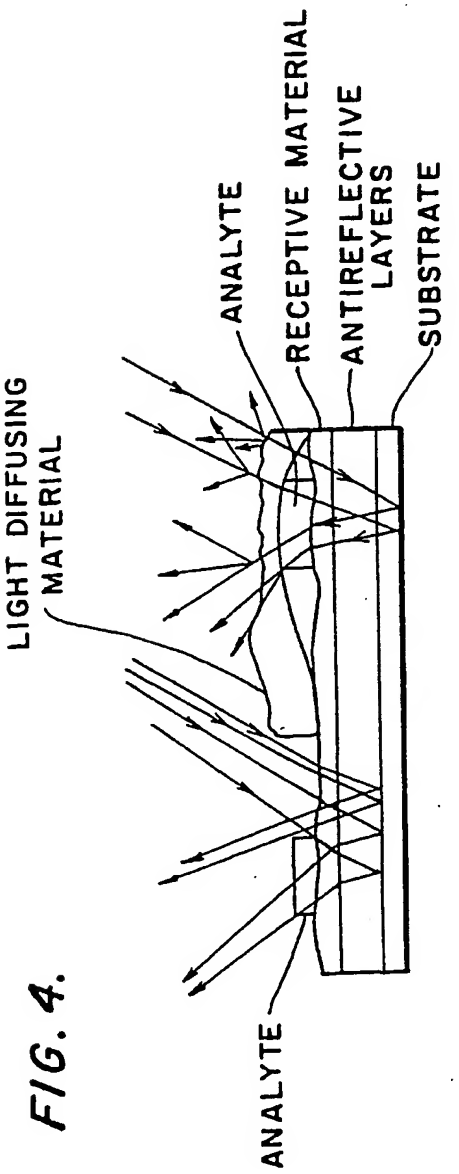
LIGHT DIFFUSING MATERIAL
ANALYTE
RECEPTIVE MATERIAL
ANTIREFLECTIVE LAYERS
SUBSTRATE
ANALYTE

FIG. 4.

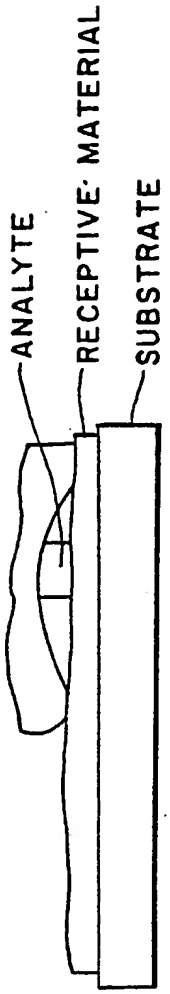
ANALYTE
RECEPTIVE MATERIAL
SUBSTRATE

FIG. 5.

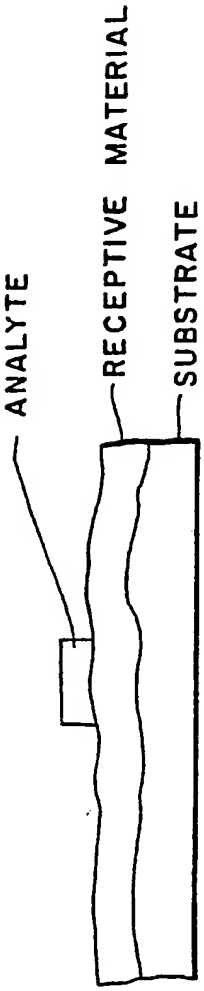
ANALYTE
RECEPTIVE MATERIAL
SUBSTRATE

FIG. 6.

INTERNATIONAL SEARCH REPORT

International Application No PCT/US90/05316

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): G01N 21/00,21/75,33/552,33/553

U.S.Cl.: 436/164,170,525,527,805; 422/56,57,58; 356/301

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
U.S.	436/164,169,170,518,525,527,528,531,805,807; 422/56,57,58; 356/301,317,318,322; 250/461.2

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
Y	US, A, 4,054,646 (GIAEVER) 18 October 1977, see entire disclosure.	1-37
Y	US, A, 4,090,849 (HEALY ET AL.) 23 May 1978, see entire disclosure.	1-37
Y	US, A, 4,558,012 (NYGREN ET AL.) 10 December 1985, see entire disclosure.	1-37
Y	US, A, 4,647,544 (NICOLI ET AL.) 03 March 1987, see entire disclosure.	1-37
A	US, A, 4,654,300 (ZUK ET AL.) 31 March 1987, see the abstract.	18-22
A	US, A, 4,737,464 (MCCONNELL ET AL.) 12 April 1988, see the abstract.	18-22
Y	US, A, 4,820,649 (KAWAGUCHI ET AL.) 11 April 1989, see entire disclosure.	1-37

* Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]: 17 December 1990

Date of Mailing of this International Search Report [2]: 06FEB 1991

International Searching Authority [1]: ISA/US

Signature of Authorized Officer [20]: Kimberly A. Trautman

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No [18]
A	Chemical Abstracts, Vol. 78, issued 1973, Erdman et al., "Etching solutions for germanium and silicon", see page 438, Ger. Offen. 2,245,809, abstract no. 166025X.	1-37
A	Biosensors, Volume 1, 1985, J. F. Place et al., "Opto-Electronic Immunosensors: A Review of Optical Immunoassay At Continuous Surfaces", pages 321-353. See the abstract.	1-37
Y	Journal of Applied Optics, Volume 24, No. 4, 15 February 1985, T. Sandström et al., "Visual Detection of Organic Monomolecular Films by Interference Colors", pages 472-479. See entire document.	1-37
Y	Photonics Spectra, February 1988, J. Hanlin, "Thin Films: New Medical Detectives", see entire document.	1-37
A	Electrophenomena, Vol. 98, issued 1983, T. STOEV, "A study on the Physics and Technology of VMOS Structures", Bulg. J. Phys. 9(3), pages 277-284. See the abstract.	2-6,32,36
A	J. Electranal. Chem, Vol. 75, 1977, M. W. Humphreys, "Ellipsometry of DNA Adsorbed at Mercury Electrodes, A Preliminary Study", pages 427-436. See the abstract.	1-37

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

Y,P	US, A, 4,921,878 (ROTHMAN FT AL.) 01 May 1990, see the abstract.	2-6,10,32, 36
Y,P	US, A, 4,931,384 (LAYTON ET AL.) 05 June 1990, see entire disclosure.	1-37
A	Chemical Abstracts, Vol. 96, issued 1982, Fujitsu Ltd:, "Etching", see page 743, JP, B, 81,144541 abstract No. 96:96322N.	1-37

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers , because they relate to subject matter [1] not required to be searched by this Authority, namely:

2. ☐ Claim numbers , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [1], specifically:

3. ☐ Claim numbers_____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.